Ice Nucleation, Propagation, and Deep Supercooling in Woody Plants

M. Wisniewski[a]; M. Fuller[b]; J. Palta[c]; J. Carter[d]; R. Arora[e]

[a] U.S. Department of Agriculture, Agricultural Research Service, Appalachian Fruit Research Station, Kearneysvile, WV, USA [b] University of Plymouth, Newton Abbot, UK [c] Department of Horticulture, University of Wisconsin, Madison, WA, USA [d] Department of Horticulture, University of Minnesota, St. Paul, MN, USA [e] Department of Horticulture, Iowa State University, Ames, IA, USA

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M. Wisniewski
M. Fuller
J. Palta
J. Carter
R. Arora

SUMMARY. The response of woody plants to freezing temperatures is complex. Species vary greatly in their ability to survive freezing temperatures and the resulting dehydrative and mechanical stresses that occur as a result from the presence of ice. Initially, this is presented by the ability to inhibit the formation of ice (ice nucleation) by supercooling. Significant questions exist about the role of internal and external ice nucleating

M. Wisniewski is Plant Physiologist, U.S. Department of Agriculture, Agricultural Research Service, Appalachian Fruit Research Station, Kearneysville, WV 25430 (E-mail: mwisniew@afrs.ars.usda.gov).

M. Fuller is affiliated with Seale-Hayne Faculty of Agriculture, University of Plymouth, Newton Abbot, UK.

J. Palta is affiliated with the Department of Horticulture, University of Wisconsin, Madison, WI 53706.

J. Carter is affiliated with the Department of Horticulture, University of Minnesota, St. Paul, MN 55108.

R. Arora is affiliated with the Department of Horticulture, Iowa State University, Ames, IA 50011.

agents in determining the extent to which any particular plant can supercool. Additionally, little is known about how plant structure can affect ice nucleation and propagation. In this review, the ability of high-resolution infrared thermography to reveal significant details about the freezing process is demonstrated. In general, the presence of effective, intrinsic nucleators appear to be common in woody plants. The nucleators appear to be as effective as external ice nucleators and induce stems to freeze at warm, subzero temperatures. Barriers appear to exist, however, that prevent ice propagation into lateral appendages such as buds, or newly extended primary tissues. Deep supercooling represents a unique adaptation of woody plants to avoid freezing injury by dramatically suppressing ice formation in specific tissues. The extent of suppression is limited by the homogeneous nucleation temperature of water ($-38°C$) and therefore deep supercooling is characteristic of moderately hardy woody plants. In contrast, it has been proposed that the most cold-hardy woody plants have the ability to form glassed solutions. These solutions are very stable as long as the cell remains below the melting temperature of the glass and so allows tissues to become relatively impervious to the stresses associated with extremely low temperatures. *[Article copies available for a fee from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: <docdelivery@haworthpress.com> Website: <http://www. HaworthPress.com> © 2004 by The Haworth Press, Inc. All rights reserved.]*

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INTRODUCTION

In the course of a year, perennial plants in temperate climates are exposed to several types of freezing stress including low temperature extremes and unseasonable episodes of frost. In response to short daylength and low temperature, woody plants cold acclimate (Wisniewski and Arora, 1993; Sakai and Larcher, 1987; Artlip and Wisniewski, 2001). This is a dynamic process that not only exhibits a distinct seasonality but the mechanisms that confer cold hardiness in midwinter may differ from those in late winter or early spring. This is further complicated by the fact that in many perennial woody plants can respond very differently to freezing temperatures.

Freezing injury can occur in plants only after the formation of ice within their tissues. Knowledge of the patterns of ice nucleation and propagation is important to understanding freeze-stress-resistance

mechanisms in plants (Workmaster et al., 1999). While ice formation in plants has been studied by thermal analyses using thermocouples, this technique does not yield direct data about either the location of ice initiation or the temperature at that specific time point. In contrast, infrared thermography has recently been used to visualize ice nucleation and propagation in plants (Wisniewski et al., 1997; Wisniewski and Fuller, 1999; Wisniewski et al., 2001; Fuller and Wisniewski, 1998; Pearce and Fuller, 2001). This technique allows for the direct visualization of the freezing process in intact plants under both laboratory and field conditions so one can obtain data on the number and location of initial freezing events, whether or not they occurred on the surface or internally, the temperature at which ice formed, and the direction and rate of ice propagation. Data from these studies relevant to ice formation in woody plants will be discussed.

Also unique to the buds and xylem tissues of several species of temperate woody plants is the ability to avoid freezing stress by deep supercooling. A comprehensive list of woody species that exhibit this trait has been presented by George et al. (1982). During a deep supercooling event, cellular water remains liquid within cells at very low temperatures by remaining isolated from heterogeneous ice nuclei and the nucleating effect of extracellular ice. Supercooled water is in a metastable condition and will form intracellular ice in response to a heterogeneous nucleation event or when the homogeneous nucleation temperature ($-38°C$) of water is reached. A general review of this topic will also be presented.

ICE NUCLEATION AND PROPAGATION

The subject of ice nucleation in plants has been comprehensively reviewed by Ashworth and Kieft (1995). Ice formation can be induced by either intrinsic or extrinsic nucleating sources. Once ice has been initiated, barriers and avenues to ice propagation, both within the plant and at the plant surface, determine the subsequent pattern of ice formation and are a factor in the survival mechanism exhibited by a given plant tissue or organ. Barriers to ice propagation within a plant can be permanent or temporary (Workmaster et al., 1999), i.e., they can prevent or just delay the growth of ice crystals. Some barriers may also only be present at particular times of the year, or upon completion of particular stages of development. The inability of ice to propagate into buds, due to a lack of differentiated vascular tissue (Ashworth, 1982; Quamme et

al., 1995) is an example of such a temporal barrier. In wheat (*Triticum aestivum* L.) ice propagation was impeded for several hours at stem and rachis nodes (Single, 1964).

Wisniewski et al. (1997) demonstrated in a comprehensive manner that infrared thermography could reveal details about the freezing process that could not be easily obtained with the use of thermocouples. Using this technology, they were able to demonstrate the freezing of water droplets on the surface of plants occurring as independent events, and that droplets containing populations of the ice-nucleating-active bacterium, *Pseudomonas syringae*, would freeze at warmer temperatures than plain water alone. The presence of these water droplets could induce open flowers of peach (*Prunus persica* L. Batsch) and apple (*Malus domestica* L.) to freeze. However, the presence of efficient intrinsic nucleators, that were active at warm sub-zero temperatures, in these plants was also documented. In most cases, ice would be initiated in the outer bark of stem tissues and then propagate into expanded primary tissues such as flower buds or young shoots, despite the fact that these tissues were supercooled to lower temperatures (due to radiative cooling) than the stem tissues themselves. An example of ice propagation in a stem of peach is illustrated in Figure 1. Ice was initiated in the bark tissues and progressed more rapidly around the stem than across it. Importantly, this study also demonstrated that thick leaf cuticles could also act as a barrier to ice propagation. Water droplets frozen on the surface of leaves of rhododendron (*Rhododendron* sp.) could not induce the leaves to freeze. Rather, ice formation was initiated inside the stem and propagated into the lateral appendages (Figure 2). Preliminary studies have indicated that infrared thermography could also be used to study the freezing process in conifers (Sutinen et al., 2001).

In a detailed study of the freezing of cranberry (*Vaccinium macrocarpon* Ait.) uprights and fruit, Workmaster et al. (1999) used infrared thermography to reveal unique details about the freezing process. Their results indicated that leaves were nucleated by ice penetration via stomata located on the abaxial surface and that the thick cuticle present on the adaxial surface was an effective barrier to extrinsic nucleation. Furthermore, cranberry fruit have the ability to supercool due to the presence of barriers present at both the pedicel and fruit surface. Only frozen moisture present at the calyx end of the fruit in the remnant nectary could induce the fruit to freeze, most likely through stomata. Leaves only froze when ice was present on the abaxial surface. Once initiated, ice propagated to the stem and then readily to other leaves. In both unripe and ripe fruit, ice propagation from the stem to the fruit was never observed

FIGURE 1. Infrared video images of ice propagation across a stem section of peach. Ice formation, visible as light area, was initiated in the outer bark tissues (A) and then slowly propagated across the stem (B and C).
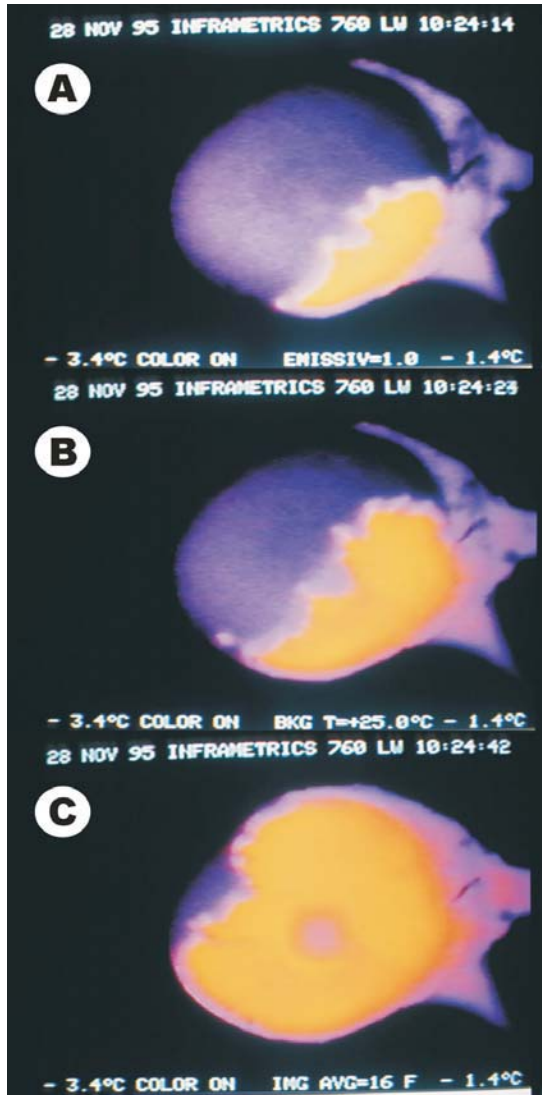
FIGURE 2. Infrared video images of ice nucleation and propagation in a detached shoot section of rhododendron cv. Olga. A frozen droplet of water containing ice-nucleating active bacteria can be seen as a bright spot on the surface of an upper leaf. Despite the presence of the frozen droplet, ice formation was initiated internally in a lower portion of the stem. In (A), the upper leaflets have already begun to freeze while the two lowest visible leaves have not begun to freeze. The freezing event can be seen as a warming of the leaf tissue due to the heat of fusion that is released when water undergoes a phase transition from a liquid to a solid. The camera temperature range is presented at the bottom of each picture. Brighter colors represent warmer temperatures while the darker colors represent colder temperatures within the set temperature range. In B, ice can be seen propagating in the mid vein of the lower leaf (arrow). In C and D, all leaves have frozen and the heat released during the freezing process is beginning to dissipate and the leaf temperature is beginning to approach the ambient, background temperature.
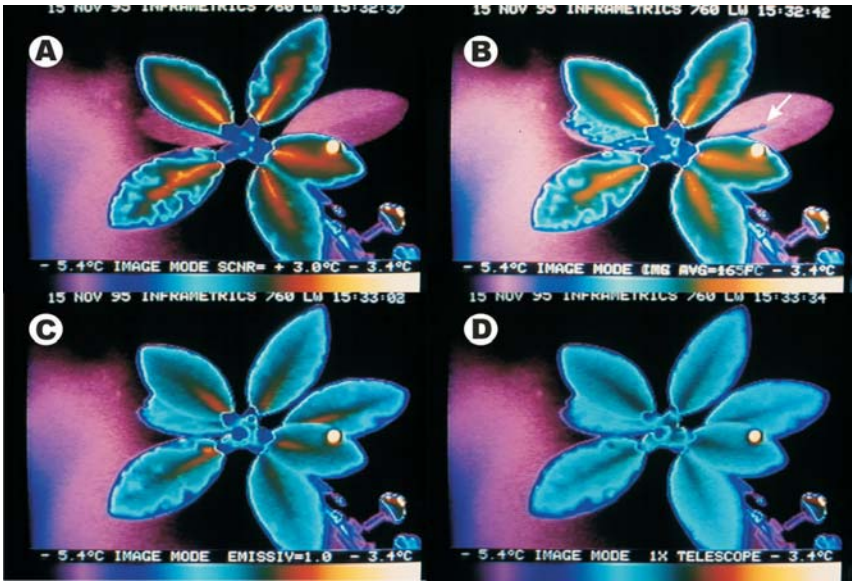
(Figure 3). Fruit could remain supercooled for up to one hour (the duration of the experiment). Ripe berries supercooled to colder temperatures and for longer durations than unripe berries.

Carter et al. (1999, 2001) have also used infrared thermography to study the freezing process in flowering shoots of blackcurrant (*Ribes nigrum* L.). In the initial study (Carter et al., 1999), it was observed that differences in field ratings of flower cold hardiness of cultivars could

FIGURE 3. Ice propagation in fruit-bearing, cranberry uprights. Ice can be seen propagating in the stem of the upright on the right. Despite propagation of ice throughout the stem and leaves, ice did not propagate through the pedicel into the stem. This provides evidence that some type of barrier exists that prevents ice propagation through the pedicel and thus allows the fruit to supercool.
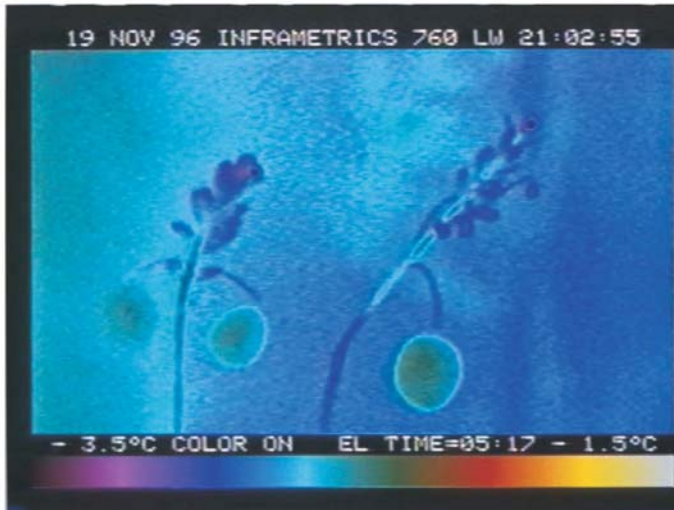
not be correlated with differences in flower bud hardiness obtained in laboratory freezing studies. This indicated that perhaps the flowering strigs of blackcurrant were supercooling. This was supported by the observation that ice nucleation in stem tissues occurred at around $-2°C$ while detached flowers could supercool to $-9°C$. The patterns of freezing injury in shoots with flowering strigs frozen to $-5°C$ also indicated the possibility that barriers to ice propagation from stem tissue to the raceme may exist and play an important role in the mechanism of frost resistance. In the subsequent study (Carter et al., 2001), patterns of ice formation and movement were studied. The pattern was discerned to be quite complex. The stem tissues of blackcurrant were always the first to freeze, however, it was common to see several independent movements of ice into the peduncle of a raceme and that individual flowers attached to the same peduncle could also freeze independently. It was concluded that barriers to movement of ice exist at specific anatomical junctions within the plant, notably where the peduncle of an inflorescence attaches to a stem and where a flower pedicel joins a peduncle. The time re-

quired for ice to pass through these barriers was inversely related to the degree of supercooling that had occurred prior to freezing.

The above mentioned studies clearly indicate that barriers to ice nucleation and propagation exist in plants and can play an important role in frost resistance. Furthermore, they indicate that the creation and/or enhancement of barriers could be used to improve frost protection. Such an approach could involve both selection for specific anatomical or structural traits or the physical application of materials that provide a barrier to ice nucleation. The latter approach has been recently explored in herbaceous plants (Wisniewski et al., 2002). In this study, an application of a hydrophobic particle film was administered to tomato plants (*Lycopersicon esculentum* L.) prior to the application of water containing ice-nucleation-active bacteria. In studies conducted in an environmental chamber, noncoated plants froze at temperatures around $-2.5°C$, while coated plants exhibited the ability to supercool to temperatures as low as $-6.0°C$. Studies conducted in an environmental chamber specifically designed to simulate radiative frost conditions also resulted in significant differences between coated and noncoated plants. When water was sprayed on the plants to simulate dew, it collected on noncoated plants but did not collect on the plants coated with the hydrophobic particle film (Figure 4). The water present on the surface of the noncoated plants resulted in leaf temperatures lower than chamber temperatures due to evaporative cooling. This is seen as large black areas on the noncoated plants illustrated in Figure 4.

DEEP SUPERCOOLING AND THE FORMATION OF GLASSES

Deep supercooling of flower buds and xylem tissues, in which water in cells or tissues remain isolated from the nucleating and dehydrative effects of extracellular ice, has been comprehensively reviewed (Ashworth, 1992; Wisniewski and Arora, 1993; Wisniewski, 1995; Wisniewski and Fuller, 1999; Wisniewski and Arora, 2000). Supercooling of floral buds stands in contrast to extraorgan freezing where water migrates to sites of extracellular ice away from the bud primordia (Sakai and Larcher, 1987) and equilibrium freezing in bark and xylem tissues where cells lose water to ice crystals present in the surrounding extracellular space (Chen et al., 1995). In both cases, the amount of water that is lost from a cell is directly dependent on the vapor pressure (and hence temperature) of the ice in the surrounding tissue. The lower the temperature the more cellular water is displaced. As a result, the concentration of solutes present in

FIGURE 4. Tomato plants either coated with a hydrophobic particle film (left) or non-coated (right). Both plants were sprayed with water and then placed in an environmental chamber and cooled. Water on the non-coated plant can be seen as large dark patches that represent temperatures outside of the set temperature range (-0.2 to $1.7°C$). The presence of water resulted in lower leaf temperatures due to evaporative cooling. Water did not collect on the coated plants.
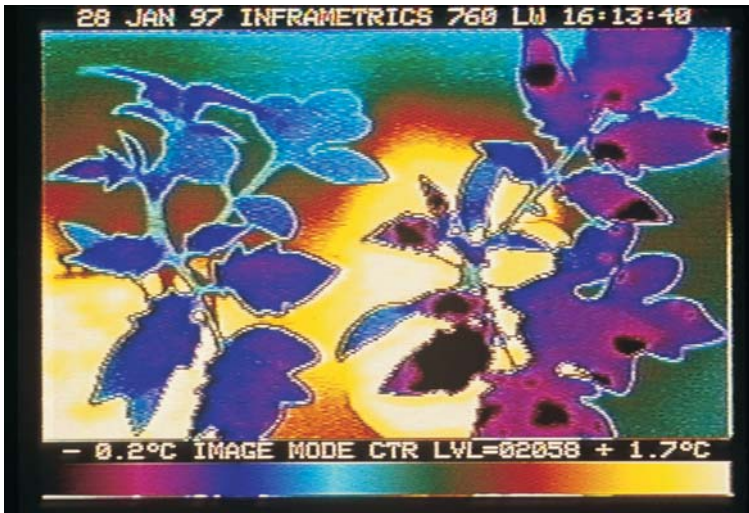
the cell increases and the freezing point decreases. Generally speaking, the major stress experienced by frozen tissues is believed to be severe dehydration and the cellular changes (membrane folding, protein denaturation, increased level of toxic solutes) that result (Artlip and Wisniewski, 2001; Chen et al., 1995; Wisniewski and Arora, 2000). In the present review, only deep supercooling of xylem tissues will be discussed and the reader is referred to other reviews for details on deep supercooling of floral buds vs. extraorgan freezing.

Of the many aspects of plant cold hardiness, deep supercooling is perhaps the most enigmatic (Wisniewski and Arora, 2000). The ability of some plants to maintain symplastic water in an unfrozen condition and without movement of the water into the apoplast is a remarkable adaptation that has not failed to impress both biophysicists and plant physiologists. Although the ability of woody plant tissues to avoid freezing by deep supercooling was first suggested in the 1960s (see review by

Wisniewski, 1995), the mechanism that allows small domains of water to avoid freezing, despite the presence of extracellular ice, remains little understood. This partly is due to the fact that the properties which allow deep supercooling to occur apparently rely on the structural organization of the tissue or organ. This feature has made it very difficult to manipulate plant material in a way to discover the fundamental mechanism and/or properties that allow deep supercooling to occur.

Deep supercooling of xylem tissues is a common characteristic of many temperate species of woody plants. Using differential thermal analysis (DTA), one can observe freezing events occurring in xylem tissues at very low temperatures (-25 to $-40°C$) whose appearance is correlated with death of the tissue (Wisniewski and Arora, 1993). Because of this association, DTA has been used extensively to evaluate the degree of cold hardiness of stem tissues of many important species of fruit and landscape species of woody plants. In order for deep supercooling to occur, a barrier must exist in xylem tissues that prevents the rapid loss of water to extracellular ice and also prevents the growth of ice crystals into living cells. In this regard, the porosity and/or permeability of the cell wall appears to play an essential role in the regulation of deep supercooling. In a series of papers (Wisniewski and Davis, 1989; Wisniewski et al., 1991a; Wisniewski et al., 1991b), it was demonstrated that the structure and composition of the pit membrane of xylem parenchyma cells appear to play an important role in regulating the extent of deep supercooling of xylem tissues. The pit membrane is a thin portion of the cell wall that allows for the passage of solutes and other materials, including plasmodesmatal connections, between cells. It is composed mainly of cellulose and pectic materials and unlike secondary wall material is unglignified for at least two years of development. By altering (chemically or enzymatically) the compostion of this layer of the cell wall, it was demonstrated that the extent of deep supercooling could be reduced or eliminated. In particular, the pectic component of the pit membrane appeared to play a key role in regulating its porosity, although a unique arabinogalactan-rich glycoprotein was also identified in the amorphous layer subtending the pit membrane. The role of this protein is unknown.

Another unique response of cells in woody plants is the ability to form glassed cell solutions (Chen et al., 1995; Hirsh et al., 1985). This believed to be a natural adaptation that occurs in extremely hardy plant species that can survive cooling to $-196°C$ in liquid nitrogen. The formation of glasses is a unique metastable condition that cannot be terminated below the glass transition temperature. Aqueous glasses are

extremely viscous brought about by high solute (sugar) concentrations at a sufficiently low temperature. In poplar (*Populus tremuloides*), glasses can form below $-20°C$ (Hirsh et al., 1985). Although glassed solutions are extremely metastable and under a high degree of supercooling and hydrostatic tension, they are not subject to ice nucleation, solute crystallization, or water vapor cavitation so long as the solution remains below the melting temperature of the glass. Thus, the cytoplasm and its contents are extremely stable and relatively unaffected by the stresses associated with low temperature and the presence of ice. The glassed state is frequently relied upon for the survival of plant tissues during cryopreservation (Steponkus et al., 1992).

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